Exopolysaccharides Synthesized by *Lactobacillus reuteri* Protect against Enterotoxigenic *Escherichia coli* in Piglets

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Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhea in piglets; ETEC cells colonize the intestinal mucosa with adhesins and deliver toxins that cause fluid loss. This study determined the antiadhesive properties of bacterial exopolysaccharides (reuteran and levan) and related glycans (dextran and inulin) in a small intestinal segment perfusion (SISP) model. The SISP model used 10 jejunal segments from 5-week-old piglets. Five segments were infected with ETEC expressing K88 fimbriae (ETEC K88), while five segments were treated with saline. Every two segments (ETEC and non-ETEC infected) were infused with 65 ml of 10 g liter⁻¹ of glycans or saline (control) for 8 h. High-resolution melting-curve (HRM) quantitative PCR (qPCR) indicated that *E. coli* is the dominant bacterium in infected segments, while other bacteria were predominant in noninfected segments. Infection by ETEC K88 was also verified by qPCR; gene copy numbers of K88 fimbriae and the heat-labile toxin (LT) in mucosal scrapings and outflow fluid of infected segments were significantly higher than those in noninfected segments. Genes coding for K88 fimbriae and LT were also detected in noninfected segments. LT amplicons from infected and noninfected segments were 99% identical over 481 bp, demonstrating the presence of autochthonous ETEC K88. All glycans reduced fluid loss caused by ETEC K88 infection. Reuteran tended ($P = 0.06$) to decrease ETEC K88 levels in mucosal scraping sample, as judged by qPCR. Fluorescent *in situ* hybridization analysis demonstrated that reuteran significantly ($P = 0.012$) decreased levels of adherent ETEC K88. Overall, reuteran may prevent piglet diarrhea by reducing adhesion of ETEC K88.

Enterotoxigenic *Escherichia coli* (ETEC) infects humans and animals. ETEC infection is a major cause of traveler's and infant diarrhea (1, 2). ETEC also infects young farm animals such as calves and piglets (3), leading to economic losses due to mortality, morbidity, decreased growth rates, and cost of medication (4). ETEC colonizes the small intestine by fimbriae that attach to intestinal epithelial glycoprotein receptors (5). The subsequent secretion of heat-stable and/or heat-labile enterotoxins leads to a disruption of electrolyte balance and diarrhea (6–8). Adhesion to mucosal tissue is host specific. Human ETEC strains adhere with fimbriae termed colonization factor antigen I and coli surface antigens 1 to 6 (9). Porcine strains of ETEC adhere with K88 (F4) and F18 fimbriae; strains expressing K88 and F18 fimbriae account for 93% of ETEC infections in piglets (10, 11).

Antimicrobial growth promoters or therapeutic antibiotics are widely used to prevent or treat diarrhea of farm animals (12). The use of antibiotics, however, supports the development of antibiotic resistance (12). This resistance leads to more severe postweaning syndromes (13) and contributes to the transfer of antibiotic resistance to human pathogens (13). Therefore, alternatives are needed to control ETEC in swine. The multitude of colonization factors expressed by ETEC complicates vaccine development (14). Feed supplementation with egg yolk antibodies from chickens immunized with K88 or F18 adhesins, dietary organic acids, phage therapy, or the use of probiotics may allow the control of ETEC in pig production (15). The inhibition of bacterial adherence via receptor analogs of epithelial glycoprotein is a promising method to prevent ETEC infection (16). Inulin and fructooligosaccharides reduced the adherence of enteropathogenic *E. coli* to Caco-2 and Hep-2 tissue culture cells (17). Polysaccharides from naturally fermented green olive brines reduced the adhesion of ETEC expressing K88 fimbriae (ETEC K88) to IPEC-J2 cell lines (18). Reuteran and levan produced by *Lactobacillus reuteri* strains

TMW1.656 and LTH5794 prevented hemagglutination by ETEC K88 (19). However, only a few *in vivo* studies indicated that selected glycans decrease illness incidence in piglets. For example, dietary mannan oligosaccharides promoted the growth of nursery piglets in three farms (20), and chitoooligosaccharide increased the growth of weaning piglets and decreased the incidence of diarrhea (21). The limited number of animal studies, however, hampers practical application of dietary glycans to prevent pathogen adhesion. Moreover, prevention of pathogen adhesion has not been demonstrated as a mechanism that was responsible for the beneficial effects observed *in vivo*.

It was therefore the aim of this study to determine whether reuteran and levan, exopolysaccharides (EPSs) that prevented adhesion of ETEC to porcine erythrocytes (19), also reduce pathogen adhesion and ETEC-induced diarrhea in swine. The piglet small intestinal segment perfusion (SISP) model was used to quantify adhesion of ETEC as well as ETEC-induced fluid accumulation. The SISP model has been developed to quantify the fluid and electrolyte losses caused by infection with diarrheal pathogens (22–24). The model may also be suitable for investigation of the potential role of dietary glycans in the prevention of pathogen adhesion *in vivo*. The SISP model maintains the blood

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supply of the intestinal segments and allows sampling over time, and each animal can be used as its own control. To confirm that the antidiarrheal effects are attributable to the prevention of pathogen adhesion, microbiota in the intestinal segments were characterized by quantitative PCR (qPCR), high-resolution melting-curve qPCR (HRM-qPCR), and fluorescent *in situ* hybridization (FISH).

MATERIALS AND METHODS

Strains and culture conditions. Two ETEC strains carrying the K88 antigen were obtained from the *Escherichia coli* Laboratory at the University of Montréal. *E. coli* strains ECL13795 (O149; virotype STb:LT:EAST1:F4) and ECL13998 (O149; virotype STa:STb:LT:EAST1:F4:Paa) were cultivated on Minca agar (25) at 37°C overnight. Strain identity was confirmed by PCR targeting the genes for K88 fimbriae, heat-labile toxin (LT), and heat-stable toxin (ST). Cell suspensions from Minca agar were characterized with respect to the optical density at 600 nm (OD_{600}) and cell counts to establish that an OD_{600} of 1.2 corresponds to a cell count of 10^9 CFU ml^{-1} for both strains. Exopolysaccharide-producing *L. reuteri* strains TMW1.656 and LTH5794 were cultivated overnight at 37°C on modified DeMan-Rogosa-Sharpe (mMRS) agar containing 100 g $liter^{-1}$ sucrose as the sole carbon source. Single colonies were subcultured in 10 ml sucrose-mMRS broth for inoculation of 1 liter sucrose-mMRS broth and incubation overnight.

Purification of exopolysaccharides. Reuteran produced by *L. reuteri* TMW1.656 and levan produced by *L. reuteri* LTH5794 were harvested via ethanol precipitation and purified by dialysis and hot-phenol extraction, as described previously (19). In brief, the culture supernatant from 1 liter of cultures grown overnight in sucrose-mMRS broth was obtained by centrifugation, and 2 volumes of chilled ethanol was added to the supernatant. Exopolysaccharides were precipitated overnight at 4°C, harvested by centrifugation, and dialyzed by using membrane tubing with a molecular weight cutoff of 12,000 to 14,000 (Spectra/Por 2 membrane tubing; Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) at 4°C for 3 days. The retentate was freeze-dried, redissolved in Milli-Q water to a concentration of 20 g $liter^{-1}$, and mixed with an equal volume of Ultra-Pure buffer-saturated phenol (Invitrogen, Burlington, ON, Canada). The mixture was incubated in a water bath at 70°C for 70 min, cooled on ice for at least 30 min, and centrifuged at $3,000 \times g$ for 20 min at 4°C. The aqueous layer was collected and dialyzed as described above for 4 days to remove phenol. Samples were freeze-dried and kept at $-20^{\circ}C$. Dextran from *Leuconostoc mesenteroides* and inulin from chicory were obtained from Sigma-Aldrich (Oakville, ON, Canada).

Hemagglutination. Cultures of *E. coli* ECL13795 and ECL13998 grown overnight on Minca agar (25) were washed with 1.5 ml of phosphate-buffered saline (PBS) (pH 7.2). Hemagglutination tests were conducted as described previously (19). Twenty-five-microliter ETEC suspensions were diluted 2-fold in V-bottom 96-well polystyrene microtiter plates (Corning). The same volume of PBS or PBS containing EPS at a concentration of 10 g $liter^{-1}$ was added, and the suspensions were incubated for 5 min with gentle shaking. Erythrocytes were harvested by washing porcine whole blood (Innovative Research, Novi, MI, USA) three times in PBS and suspended in PBS at 5% (vol/vol). Twenty-five microliters of the 5% erythrocyte suspension was applied to each well. Microtiter plates were mixed gently and visually inspected after 2 h of incubation at 4°C. Triplicate independent replicates were conducted.

Animals and surgical procedure. All animal procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care and Use Committee: Livestock of the University of Alberta. Six crossbred weanling gilts (Duroc $\times$ Large White/Landrace F_1 , aged 5 to 6 weeks, with a body weight of 10.2 ± 0.7 kg) with no history of gastrointestinal disturbances were randomly selected for use in the SISP model (24). Pigs were group housed and provided with free access to a commercial

starter diet until 16 h before surgery, when pigs were isolated and fasted overnight.

Prior to the surgical procedure, pigs were tranquilized. Once a sufficient depth of anesthesia was reached, pigs were intubated and remained under general anesthesia (4% [vol/vol] isoflurane; Abbott Animal Health, Abbott Park, IL, USA) and oxygen (5 to 10 $ml\ kg^{-1}\ min^{-1}\ O_2$) for the duration of the experiment. The surgical site was cleaned, a midline incision was created, and the underlying mesentery was removed to expose the intestine. An incision was made across the intestine without cutting the mesentery; a second incision was made 20 cm distal to the first incision, again keeping the mesentery intact. This 20-cm section of intestine served as the first segment; nine additional 20-cm segments were created along the median 50% of the jejunum. Between each pair of odd and even segments, a 2-cm segment was created for later measurement of intestinal circumference. Inflow tubes and outflow tubes were attached to each intestinal segment. Inflow tubes were connected to 100-ml syringes containing the assigned treatments, and outflow tubes were connected to drainage bottles for collection of net fluid losses.

Experimental design and sample collection. Standardized cell suspensions of *E. coli* ECL13795 were obtained by washing cultures grown overnight on Minca plates with PBS; the OD_{600} of cell suspensions was adjusted to 1.2. Five milliliters of the ETEC cell suspension was introduced into the lumen of odd segments, and 5 ml PBS was introduced into even segments as pair-matching segments. Fifteen minutes after injection, perfusion into segments was conducted with 64 ml PBS (pH 6.6) containing reuteran, levan, dextran, or inulin at 10 g $liter^{-1}$ each or saline (control) for 8 h. Glycans were allotted to 4 pairs (reuteran, levan, dextran, or inulin) of ETEC-infected and control segments in a 4-by-4 Latin square to ensure that each glycan was rotated in different segment pairs among four piglets. Nonabsorbed fluid from each segment was collected into the drainage bottles. At the end of the experiment, the pig was killed, segments were opened longitudinally, and tissue samples were collected for analysis of morphology and ETEC counts. Morphological samples with an area of 0.5 to 1 cm^2 were transferred into a chilled Carnoy solution (ethanol-acetic acid-chloroform, 6:6:1) immediately and stored at 4°C for later assessment. Mucosal scraping samples for DNA extraction were obtained by scraping the surface area of intestinal segments (8 to 9 cm by 4 to 5 cm) with a glass slide. Scraping samples were flash frozen in liquid nitrogen immediately after collection and stored at $-80^{\circ}C$. Prior to DNA extraction, samples were thawed, weighed, and suspended in 1 ml TN150 buffer (10 $mmol\ liter^{-1}$ Tris, 150 $mmol\ liter^{-1}$ NaCl, pH 8.0). Outflow fluid samples were stored at $-20^{\circ}C$.

Genomic DNA extraction and quantitative PCR analysis of intestinal bacteria. Bacterial DNA was extracted from mucosal scraping and outflow fluid samples by using a DNA Stool minikit and a DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA, USA), respectively. DNA concentration and quality were checked on a NanoDrop ND-2000 spectrophotometer system (Fisher Scientific, Wilmington, DE, USA). Quantitative PCR was performed on a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) with primers targeting the eubacterial 16S rRNA genes or genes coding for K88 fimbriae and LT (Table 1). To generate standard curves for quantification of genes, the respective genes were amplified from genomic DNA of *E. coli* with the same primer pair and purified, and the concentration was determined by UV spectroscopy. Tenfold serial dilutions of the amplicons were used to generate standard curves for absolute quantification. Standard curves for quantification of eubacteria and *E. coli* were generated with amplicons that were obtained from mucosal and outflow fluid DNA. DNA isolated from mucosal and outflow samples was diluted to a concentration of 50 $mg\ liter^{-1}$ and analyzed in duplicate in MicroAmp Fast Optical 96-well reaction plates capped with MicroAmp optical adhesive film (Applied Biosystems). The qPCR mixtures contained 12.5 μl QuantiFast SYBR green mixture (Qiagen), 0.4 mM primer, 2 μl of template DNA, and sterile Milli-Q water to a final volume of 25 μl . The cycling program included an initial denaturation step at 95°C for 5 min followed by 40 cycles of denaturation at 95°C

TABLE 1 Primers and probes used in this study

Target gene	Oligonucleotide sequence (5'–3') (name)	Product size (bp)	T_m (°C) ^a	Reference
Total bacteria	AGAGTTTGATCMTGGCTCAG (BACTf)	919	58	47
	CCGTCAATTCATTGAGTTT (BACTr)			
	CGGYCCAGACTCCTACGGG (DomainBf)	200	63	48
	TTACCGCGGCTGCTGGCAC (DomainBr)			
	Alexa546-GCTGCCTCCCGTAGGAGT (Eub338)		55	49
<i>Escherichia coli</i>	CCGATACGCTGCCAATCAGT (UspAf)	884	65	50
	ACGCAGACCGTAGGCCAGAT (UspAr)			
ETEC K88 fimbriae	GCACATGCCTGGATGACTGGTG (K88f)	439	63	51
	CGTCCGCAGAAAGTAACCCACCT (K88r)			
	Cy3-GCACATGCCTGGATGACTGGTG		55	
Heat-labile toxin	CCGTGCTGACTCTAGACCCCCA (LTf)	480	65	52
	CCTGCTAATCTGTAACCATCTCTGC (LTTr)			
	Cy5-CCGTGCTGACTCTAGACCCCCA		55	
Nonspecific binding	6FAM-ACTCCTACGGGAGGCAGC (Non338)		55	53

^a T_m , melting temperature.

for 15 s, primer annealing at optimal temperatures (Table 1) for 30 or 45 s, and extension at 72°C for 30 s. Melting curves were obtained after 40 cycles. Melting curves and agarose gel separation of amplicons were used to verify the specific amplification of target genes.

DNA sequencing. PCR amplicons obtained with primers LTf and LTTr were purified from agarose gels and sequenced by Sanger sequencing (MacroGen, Rockville, MD). Sequences were compared to each other with the Align Sequences tool of the NCBI blastn suite (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Qualitative analysis of intestinal microbiota by HRM-qPCR. High-resolution melting-curve analysis was performed on a Rotor-Gene Q instrument (Qiagen) by using Type-it HRM PCR master mix (Qiagen) containing EvaGreen fluorescent intercalating dye. Two pairs of 16S rRNA universal primers were used on DNA samples extracted from mucosal scraping and outflow fluid samples to detect adhering and luminal microbiota. The reaction mixtures contained 5 µl Type-it HRM PCR master mix, 0.7 mM primer, 1 µl of template DNA diluted to 10 mg liter⁻¹, and sterile Milli-Q water to a final volume of 10 µl. Reaction conditions included an activation step at 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, primer annealing (Table 1) for 30 s, and elongation at 72°C for 10 s. HRM analysis was carried out over a temperature range from 70°C to 95°C, increasing at 0.1°C each step, with waiting for 90 s under premelt conditions for the first step and a 2-s hold at each increment. Data analysis was performed by using Rotor-Gene Q software version 2.0.3, and figures were plotted with SigmaPlot 11.0 (Systat Software Inc., Chicago, IL, USA).

Quantification of adhering enterotoxigenic *E. coli* cells by fluorescent *in situ* hybridization. Intestine biopsy specimens (0.5 to 1 cm²) were fixed in Carnoy solution at 4°C for at least 2 days, followed by washing of biopsy specimens in fresh Carnoy solution for 1 h at 4°C, 70% ethanol for 1 h at room temperature, and 100% ethanol for 1 h at room temperature and two washes in xylene for 1 h at room temperature. The fixed intestine biopsy specimens were embedded in paraffin (Fisher Scientific, Ottawa, Canada) by using a Thermoscientific Histostar embedding machine (Fisher Scientific), sectioned at 4.0 µm by using a Leica RM 2135 sliding microtome (Leica Microsystems, Concord, Canada), and mounted on Superfrost Plus positively charged slides (Fisher Scientific). The sections were deparaffinized by passing through xylene (Leica Microsystems) (three times for 4 min each) and 100% ethanol (three times for 4 min each), followed by drying in an oven for 10 min. Lysozyme (Sigma-Aldrich) was applied to the tissue in an additional permeation step to facil-

itate detection of Gram-positive bacteria. DNA probes (Table 1) were synthesized by Integrated DNA Technologies (Coralville, USA) with fluorescent probes linked to the 5' end of the oligonucleotides. Probes (50 µl of 0.1 nM probe hybridization buffer) were added to each slide, and slides were covered with a coverslip. After hybridization for 50 min at 55°C, ProLong Gold Antifade reagent with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) was added to the tissue after removal of the coverslip by washing slides with autoclaved Milli-Q water. The sections were examined with an Axio Imager M1m microscope (Carl Zeiss Inc.), and images were captured at a ×40 magnification with an AxioCam M1m camera and AxioVision version 4.8.2.0 (Carl Zeiss Inc.). Enumeration was conducted on six fields per section by persons blind to sample coding. Bacterial cells were counted when a signal was observed with Cy5 (LT) and Alexa Fluor 546 (Alexa546) (total bacteria) but not with 6-carboxyfluorescein (6FAM) (nonsense probe) (Table 1).

Statistical analysis. Differences in bacterial populations quantified by qPCR and ETEC enumeration by FISH between treatments of the same animal ($n = 5$ or 6) were analyzed by using PROC MIXED in SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The model included the fixed effects of segment, ETEC treatment, glycan treatment, and the interaction of ETEC with glycan treatment as well as the random effect of pig. Mean separation was performed by using LSMEANS in SAS version 9.2 (SAS Institute Inc.). Results are expressed as means ± standard errors of the means. Significant differences are reported with a 5% probability of error ($P < 0.05$), and trends are reported with a 10% probability of error ($P < 0.10$).

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences reported here are [KJ416139](#) and [KJ412974](#).

RESULTS

Antiadhesive properties of reuteran and levan *in vitro*. The antiadhesive properties of exopolysaccharides from *L. reuteri* were validated by a hemagglutination assay (19). Dextran and inulin were used for comparison to reuteran and levan, respectively, because they have the same monosaccharide compositions as reuteran and levan, respectively, but differ in their linkage types. Hemagglutination of porcine erythrocytes by both ETEC strains was prevented by reuteran and to a lesser extent by levan (Table 2). Dextran and inulin did not affect hemagglutination. Equivalent results were obtained with the

TABLE 2 Inhibition of erythrocyte agglutination by two ETEC K88 strains with bacterial exopolysaccharides and structurally related polysaccharides at a concentration of 3.3 g liter⁻¹

EPS	Source	Agglutination ^a
Reuteran	<i>L. reuteri</i> TMW1.656	++
Levan	<i>L. reuteri</i> LTH5794	+
Dextran	Commercial	—
Inulin	Commercial	—

^a Stronger antiadhesive activity leads to agglutination of erythrocytes at higher ETEC concentrations; *E. coli* ECL13795 (O149; virotype STb:LT:EAST1:F4) and ECL13998 (O149; virotype STa:STb:LT:EAST1:F4:Paa) were used for detection. ++, strong agglutination observed; +, agglutination observed; —, agglutination not observed.

two ETEC K88 strains; strain ECL13795 was selected for subsequent *in vivo* experimentation.

Reduction of net fluid absorption. Antiadhesive properties of reuteran and levan with the controls dextran and inulin were evaluated *in vivo* in the SISP model. The net fluid loss was used as the primary outcome to document the antidiarrheal effect of glycans. The reduction of net fluid absorption is plotted as the difference between each infected segment and its paired noninfected segment (Fig. 1). All glycans decreased ($P < 0.05$) the net fluid loss caused by ETEC infection compared to saline (Fig. 1).

Quantification of bacteria in jejunal outflow fluid by qPCR. To determine whether the ETEC strain was infective in the SISP model, rRNA genes from eubacteria and *E. coli* and genes coding for K88 fimbriae and LT in outflow fluid were quantified by qPCR (Fig. 2). Gene copy numbers for eubacteria in fluid from infected segments ranged from 10⁸ to 10⁹ gene copies ml⁻¹. Copy numbers of *E. coli* rRNA genes and genes coding for K88 fimbriae and LT were not different from copy numbers for the total bacterial rRNA genes. In noninfected segments, total bacterial copy numbers ranged from 10⁷ to 10⁸ gene copies ml⁻¹. *E. coli* accounted for 1 to 10% of the total bacteria, and ETEC K88 accounted for 1 to 10% of *E. coli* cells (Fig. 2). Levels of eubacterial rRNA genes in infected segments were at least 10-fold higher than those in the paired noninfected segments, indicating that ETEC K88 accounted for >90% of bacterial cell counts in infected segments. Glycan infusion did not significantly influence the number of ETEC bacteria in the outflow fluid.

Analysis of jejunal luminal microbiota by HRM-qPCR. The predominance of ETEC in the lumen of infected segments was assessed by a second method, HRM-qPCR, with primers targeting eubacterial rRNA genes (DomainB primer pair). For infected segments, only one melting peak was observed (Fig. 3A), and the melting temperature of this peak matched the melting temperature of *E. coli* ECL13795, confirming the predominance of ETEC in infected segments. For noninfected segments, several peaks were observed (Fig. 3B). HRM-qPCR analysis with the Bact primer pair provided equivalent results (see Fig. S1 in the supplemental material). HRM-qPCR analysis of piglets 2, 4, 5, and 6 with the same primer pairs also gave comparable results (data not shown). Glycan infusion did not significantly influence the composition of the intestinal microbiota in the outflow fluid of infected segments.

Quantification of bacterial populations in mucosal scraping samples by qPCR. ETEC infection is initiated by attachment to mucosa, followed by the production of toxins that cause electro-

lyte imbalance and fluid loss. Therefore, bacteria in mucosal scraping samples were analyzed by qPCR to quantify attached ETEC K88. ETEC was detected in mucosal scraping samples from noninfected segments (Fig. 4), indicating the presence of autochthonous ETEC K88 in the piglets used for experiments. The number of ETEC K88 cells in noninfected segments accounted for 1 to 10% of the number of ETEC cells in infected segments. In outflow fluid, the number of ETEC bacteria in noninfected segments was <0.1% of the number of ETEC bacteria in infected segments, suggesting that the autochthonous ETEC bacteria were mucosa associated. HRM-qPCR analysis of LT amplicons demonstrated that different strains of ETEC were present in infected segments (melting temperature of 81.6°C) and noninfected segments (melting temperature of 85.4°C). LT amplicons were purified from agarose gels and sequenced. The sequences of LTs from ETEC K88 strain ECL13795 and the LT amplicon obtained from noninfected segments of piglets were 99% identical over 481 bp.

A trend toward a reduced number of adhering ETEC K88 bacteria was observed for segments infused with ETEC and reuteran ($P = 0.06$) (Fig. 4). Inulin tended to reduce gene copy numbers for K88 fimbriae ($P = 0.07$) but not gene copy numbers for LT in ETEC-infused segments (Fig. 4).

Analysis of jejunal mucosal microbiota by HRM-qPCR. Mucosal microbiota were analyzed by HRM-qPCR with the same primers employed for analysis of luminal microbiota. The predominance of *E. coli* in ETEC K88-challenged segments was confirmed by profiles of DomainB amplicons (see Fig. S2A in the supplemental material). HRM-qPCR of noninfected segments indicated the presence of bacteria other than *E. coli*, as a different melting temperature from the temperature of *E. coli* was observed to be the main melting peak for noninfected segments (see Fig. S2B in the supplemental material).

Fluorescent *in situ* hybridization. DNA isolated from mucosal scraping samples was likely contaminated by luminal, nonadhering ETEC K88, thus obscuring reduced pathogen adhesion in the presence of glycans. Evaluation of biopsy samples by FISH eliminated the contamination by luminal bacteria and visually revealed the colonization of ETEC K88 in epithelial cells. Cells

FIG 1 Reduction of net fluid absorption in piglet jejunal segments challenged with ETEC K88 or PBS and perfused with saline (control), reuteran, dextran, levan, or inulin (10 g liter⁻¹) for 8 h. Each bar represents the mean difference of the net fluid loss in ETEC-challenged segments and the net fluid loss of the paired PBS-perfused segment ($n = 6$). Mean values with unlike letters are significantly different ($P < 0.05$).

FIG 2 Quantitative PCR analysis of bacterial populations in outflow fluid samples collected from piglet jejunal segments challenged with ETEC K88 or PBS and perfused with saline (control), reuteran, dextran, levan, or inulin (10 g liter⁻¹) for 8 h. Black bars represent total bacterial numbers, gray bars represent *E. coli*, white bars represent gene copy numbers for K88 fimbriae, and hatched bars represent gene copy numbers for LT. Data are shown as means $\pm$ standard errors of the means ($n = 5$). Mean values obtained with the same primer pairs with unlike letters were significantly different ($P < 0.05$). K88, K88 fimbriae; LT, heat-labile toxin of ETEC.

were counted when their DNA hybridized to the LT and total bacterial probes but not to the nonsense probe Non338 (Fig. 5). FISH analysis confirmed that ETEC K88 attached to the porcine mucosa (Fig. 6). FISH analysis also confirmed colonization with autochthonous ETEC K88. The number of adhering ETEC cells in noninfected segments was generally >30% of the number of ETEC cells in infected segments (Fig. 6). Autochthonous ETEC K88 was thus primarily mucosa associated. Reuteran produced by *L. reuteri* TMW1.656 significantly reduced ETEC colonization of epithelial cells compared to saline. Levan from *L. reuteri* LTH5794 as well as inulin and dextran did not reduce adhesion of ETEC. In the inulin perfusion group, no difference was observed between

infected and noninfected segments but both segments had a relatively high number of ETEC cells of around 1,400/mm².

DISCUSSION

Postweaning diarrhea in piglets accounts for considerable economic losses (4). ETEC is the main cause of postweaning diarrhea in piglets (11). The use of glycans to prevent pathogen adhesion may inhibit colonization of the swine intestine by ETEC; however, *in vivo* confirmation of the efficacy of glycans and their mode of action remains scarce. The SISF model was originally developed to determine the net absorption of fluid and electrolytes in the small intestine of newly weaned pigs (24), but its use was later extended

FIG 3 Luminal microbiota melt peak profiles for pig 3 determined by the DomainB primer pair for ETEC K88-challenged (A) and non-ETEC K88-challenged (B) piglet jejunal segments after 8 h of glycan or saline perfusion. Similar profiles were obtained for all 5 animals analyzed (data not shown).

FIG 4 Quantitative PCR analysis of the genes coding for K88 (black bars) and LT (white bars) in mucosal scraping samples collected from piglet jejunal segments that were challenged with ETEC K88 or PBS and perfused with saline (control), reuteran, dextran, levan, or inulin (10 g liter⁻¹) for 8 h. Data are shown as means $\pm$ standard errors of the means ($n = 5$). Mean values (obtained with the same primer pairs) with unlike letters were significantly different ($P < 0.05$). K88, K88 fimbriae; LT, heat-labile toxin.

to evaluate the protective effects of food additives (26), nonstarch polysaccharides (27), and plant proteins (23) in ETEC-induced diarrhea. The SISF model also allowed determination of the early gene expression response of the pig small intestinal mucosa to

infection with ETEC (28) and *Salmonella* (29). Most studies employing SISF, however, focused on analysis of physiological and immune responses of the host, including fluid loss, electrolyte secretion, and the expression of cytokines (23, 26, 27, 30). Very few studies provided a detailed bacteriological analysis of the model; previous studies using the SISF model reported only cell counts of ETEC in the lumen (27, 31, 32). To determine whether glycans prevent pathogen adhesion, we focused on the quantification of mucosa-adherent ETEC. Luminal and mucosal microbiota of outflow fluid and mucosal scraping samples were characterized by qPCR and HRM-qPCR. All analyses demonstrated that ETEC ECL13795 infected the SISF model and accounted for >90% of the total microbiota in the infected segments. However, qPCR and FISH also demonstrated the presence of autochthonous ETEC K88. HRM-qPCR and sequencing excluded the possibility that ETEC K88 in noninfected segments resulted from contamination with the experimental ETEC strain ECL13795.

All glycans in this study reduced the ETEC-induced net fluid loss compared to saline. The analyses of intestinal microbiota in infected segments provided no indication that infusion with glycans altered luminal microbiota; however, in addition to the interference with pathogen adhesion, competitive exclusion or pathogen aggregation may account for the reduced fluid loss (16, 33). The presence of autochthonous ETEC reduced the sensitivity of the SISF model to detect the activity of antiadhesive glycans. In infected segments, autochthonous ETEC accounted for up to 40% of ETEC K88 cells adhering to the mucosa, and glycans may not be effective in detaching already adhering cells. Nevertheless, FISH and qPCR analyses consistently demonstrated reduced adhesion

FIG 5 Fluorescent *in situ* hybridization localization of attached ETEC K88 and total bacteria in Carnoy-fixed and paraffin-embedded SISF intestine biopsy specimens. (A to C) 5'Cy5-LT (red), 5'Alexa546-Eub338 (yellow), and 5'6FAM-Non338 (green) indicate the number and location of ETEC (A) and all bacteria (B) and nonspecific binding (C), respectively; (D) DAPI-stained nuclei and fluorescence from three probes were merged.

FIG 6 Blind enumeration of mucosa ETEC in Carnoy-fixed and paraffin-embedded SISF intestine biopsy specimens (black bars, ETEC infected; white bars, non-ETEC infected). Each bar represents the mean number of ETEC cells per square millimeter of biopsy specimen $\pm$ standard error of the mean ($n = 6$). Mean values with unlike letters were significantly different ($P < 0.05$).

of ETEC K88 to reuteran produced by *L. reuteri* TMW1.656. Because both *in vitro* data (19; this study) and the SISF *in vivo* model (this study) indicate the ability of reuteran to prevent ETEC adhesion in swine, this glycan is a promising candidate for further evaluation in feeding trials. *In vivo* data provided no indication of reduced ETEC colonization in segments perfused with levan, inulin, or dextran.

K88 fimbriae recognize specific carbohydrate structures expressed on host cell glycoprotein; terminal GlcNAc, GalNAc, and galactose moieties of enterocyte receptors interact with K88 fimbriae (34). Some dietary carbohydrates prevent pathogen adhesion, possibly by acting as receptor analogs that saturate the glycan-binding domains of bacterial lectins and thus prevent binding to host glycoproteins. For example, polysaccharides extracted from green olive fermentation brines prevented adhesion of ETEC K88 to IPEC-J2 cells (18) but not to intestinal mucus (35). Glycans evaluated *in vivo* by using the SISF model were often classified only as ethanol-soluble and ethanol-insoluble fractions derived from hydrolysis of soybean and canola meal (31) or crops (27). In our study, the structure and composition of well-defined microbial or commercial polysaccharides were tested in an ETEC-challenged SISF model at a concentration of 10 g liter⁻¹. The composition and linkage type of EPSs were reported to be important determinants for their activity in preventing pathogen adhesion (19, 33). The active reuteran is an α -(1 $\rightarrow$ 4)- and α -(1 $\rightarrow$ 6)-linked glucan, while the predominantly α -(1 $\rightarrow$ 6)-linked dextran has little or no activity (19; this study). Likewise, the β -(2 $\rightarrow$ 6)-linked levan prevents ETEC adhesion, but the β -(2 $\rightarrow$ 1)-linked inulin is less active (19). Our results indicate that the linkage type influences the antiadhesive property of polysaccharides. However, the compounds also differ in their molecular weights (dextran > reuteran; levan > inulin), and differences in the molecular weight or the degree of branching of the glycans (33) may additionally account for their different activities.

L. reuteri is recognized as an autochthonous member of the intestinal microbiota of vertebrates (36, 37). The evolution of *L. reuteri* resulted in phylogenetically distinct lineages that are adapted to their vertebrate host (38). Although *L. reuteri* has been termed a gut symbiont, the benefit of *L. reuteri* colonization to the

host is poorly understood (39, 40). *L. reuteri* TMW1.656 is closely related to the rodent-lineage *L. reuteri* strains LTH2584 and TMW1.106 (41, 42). However, *L. reuteri* also colonize the upper intestinal tract of swine (36, 43). *L. reuteri* produces exopolysaccharides *in vivo* to form the polysaccharide matrix of bacterial biofilms on nonsecretory epithelia of the upper intestine (36, 44, 45). Reuteran formed by *L. reuteri* in the upper intestine of piglets may thus prevent adhesion of pathogens in the distal intestinal tract, including the jejunum. This protective mechanism provides an identifiable benefit to the host and justifies the promotion of *L. reuteri* from “gut commensal” (45) to “gut symbiont” (39).

In conclusion, the *in vivo* SISF model used in this study confirmed the antiadhesive properties of reuteran that were previously observed *in vitro* (19). Reuteran perfusion reduced not only ETEC-induced net fluid loss but also the adhesion of ETEC K88 to the intestinal mucosa. Reuteran may be a suitable feed additive to lessen the impact of ETEC-induced diarrhea in piglets. Comparable to the commercial production of dextran from *Leuconostoc mesenteroides*, the conversion of sucrose to fructans or glucans by lactobacilli is achieved with high yields (33, 46), facilitating commercial applications of reuteran as a feed additive to prevent ETEC infections and postweaning diarrhea in piglets.

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